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ZOOLOGICAL RESEARCH METHODOLOGY

THE CAUSES OF DIFFERENCES IN THE OPTICAL DENSITY OF DENTINE LAYERS IN MAMMALIAN TEETH

G.A. Klevezal' and L.I. Sukhovskaya

Microphotometry and X-ray spectral analysis were used to study the causes of dentine layer formation. It was shown that visual estimation of the optimum optical density of the layers depends on the optical density of the neighbouring layers, i.e. that it is relative. The optical characteristics of the layers in the growth layer group (GLG) may alter both when the thickness of the section changes and when the section is immersed in a clearing medium. The optical density of the main layers within the GLG appears to be determined by the content and distribution of the mineral component of the dentine. The thin layers within the GLG are due to differences in the structure of the organic stroma of the tooth.

Ever since the suggestion was made to use the layers in the teeth of marine mammals to determine the age of these animals (Scheffer, 1950, Laws, 1952), counting the layers in the dentine or cement has

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become the generally accepted method of aging both marine and land mammals (Klevezal', Kleinenberg, 1967; Morris, 1972; Scheffer, Myrick, 1980).

The annual layer of dentine consists in the simplest case of two bands of different width and different optical density which stain with histological dyes, especially haematoxylin, with different degrees of intensity. However, each of these bands, or only the broader one, frequently includes several narrowest bands of different optical density. Hence at the International Workshop on Age Determination of Toothed Wales and Sirenians (La Jolla, USA, 1978) it was decided to replace the term "annual layer" with the term "growth layer group" (GLG), and name the bands of different optical density "layers" (Perrin, Myrick, 1980; Instructions for the Determination of the Age of Toothed Whales and Sirenians, 1980). The structure of the GLG varies from species to species and within species and may be so complex that it is difficult to count the layers in it.

It is known that dentine has an organic (collagen) base that is impregnated with calcium salts (chiefly oxyapatite) and perforated by dentine canals which are outgrowths of the odontoblasts. Practically every researcher has inquired into the causes of the formation of the layers of different optical density, but up to now no consensus has been reached either on the causes of the formation of these layers or even on the extent to which the optical density of the dentine is correlated with the degree of its calcification. For example, Nielsen-Grue (1972), using the method of microradiography, established that the more transparent layer in the dentine of Phocaena phocaena was hypercalcified relative to the less transparent one, and Hohn (1980)

using the same method of microradiography arrived at the opposite conclusion after investigating the layers of dentine of different optical density in Tursiops truncatus.

Nevertheless it is of primary importance to find out what causes p. 1408
the dentine layers comprising the GLG to form, since this knowledge will enable us to use the structure of the latter not only for determining age but also for obtaining additional information on the life history of any particular individual. That is why the list of main research tasks drawn up at the previously mentioned international workshop included making a detailed analysis of the internal structure of GLG (Perrin, Myrick, 1980). It was pointed out that it is difficult to compare the results obtained both for different species and for different parts of the same tooth due to the great variation in GLG structure.

However, differences in the results of studies of the structure of GLG may be due not only to variation in the structure itself but also to the methodological difficulties associated with such studies. The boundaries between the individual layers of dentine are often diffuse and visual estimation of the optical density depends on the type of illumination used. Moreover, during the histochemical processing of the dentine it is difficult to establish precisely how a particular layer has altered after processing if no method has been found for accurately identifying the same layers before and after processing. It is our contention that one of the most accurate methods for making such an identification is comparison of the curves characterizing the changes in optical density in the same area of dentine obtained both before and after processing under the same conditions of illumination. We shall

now describe the use of this method for studying structure of the GLG of marine mammals.

We used for this purpose teeth (from the middle of the row) of three bottle-nosed dolphins Tursiops truncatus and tusks of two Caspian seals Pusa caspica and one fur seal Callorhinus ursinus. The curves characterizing the change in optical density of dentine (characteristic curves) were obtained on an MF-4 microphotometer connected to a KSP-4 recorder. Transverse and longitudinal sections were investigated visually in transmitted and reflected light. The structure of the GLG was described, then a mark (notch) was made on the edge of the section and the latter was placed on the stage of microphotometer so that when the stage was moved the beam of light passed along the radius of the section from the notch on the edge to the centre of the tooth. The magnification of the object was X12, and the slit size (1 X 2 mm) corresponded to the image of the narrowest layer of dentine that could be distinguished by the eye. After a number of trials a recording scale of 1:10 was selected at a rate of movement of the microphotometer stage of 6 mm/min and a rate of advance of the recorder chart of 12 mm/min. The lighting conditions were kept constant.

To determine the reasons for the differences in the optical density of the different layers of the GLG we compared the characteristic curves of the same area of dentine in sections reduced in thickness from 300 to 30 μm, mounted in glycerine, subjected to partial and complete decalcification, stained with haematoxylin, and with the collagen removed. For one section we obtained a curve of the distribution of calcium and sulfur by X-ray spectral analysis on a Comeca electron probe microanalyser. We made use of several sections

of each tooth - transverse when investigating the teeth of seals, and longitudinal and transverse when investigating the teeth of bottle-nosed dolphins.

On the characteristic curve the more transparent layers of dentine were displayed as maxima and the less transparent ones as minima. The contrast between two adjacent layers was characterized by the ratio of the height of the maximum to the height of the minimum measured from 0 on the recording chart. When the flow of light passing through the section was increased the entire curve shifted upward into the region of greater transparency, while the height of the maxima and minima remained the same, although the difference between them increased. In those cases where we were interested in the changes of the contrast of the layers we measured the ratio of the heights of the maxima and minima.

The relationship between the optical density curve and the structure of the GLG as it presents itself to the eye. The characteristic curve reveals essentially the same number of more transparent and less transparent layers of dentine as can be detected by the eye under a microscope or binocular (Fig. 1, I). The first transparency maximum in the dentine corresponds to the neonatal line which separates the embryonic and postembryonic dentine. The subsequent maxima and minima characterize the changes in optical density within the GLG.

The graph shows that the absolute optical density of the p. 1409
different transparent or different opaque layers is not the same. For example, "opaque" layer 6a (Fig. 1, I, curve 150 μm) has a lower absolute optical density (i.e. is actually more transparent) than

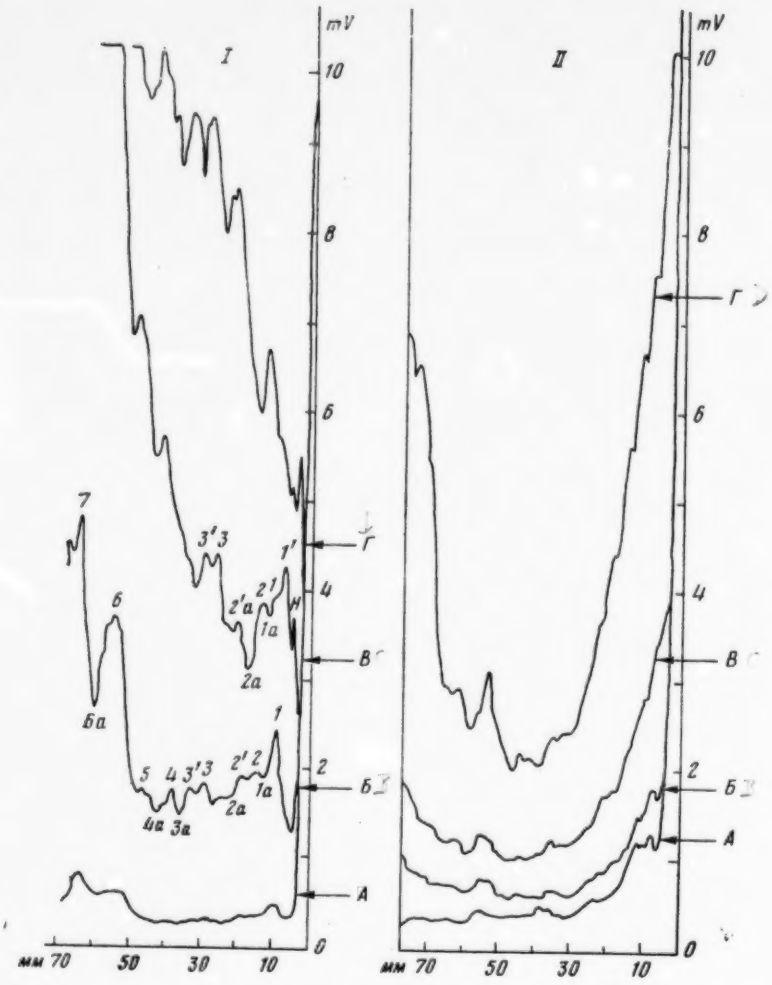

Fig. 1. Characteristic curves of the same area of a transverse section of the tooth of a Tursiops truncatus (1 - ♂, 7 GLG) and the tusk of a fur seal (II - ♀, 6 GLG) for different section thicknesses (constant illumination). I: the plain numbers and numbers with the prime symbol denote the transparent layers and the corresponding peaks on the curve; the numbers with the letter a are the opaque layers; H is the neonatal line; A is a dry section with a thickness of 310, B - 150, C - 80 μm, D is a section with a thickness of 80 μm mounted in glycerine. II: A is a dry section with a thickness of 150, B - 75, C - 40 μm, D is a section with a thickness of 40 μm mounted in glycerine.

"transparent" layer 1, whereas it looks opaque to the eye because it has next to it a layer of even lower optical density, and next to any of the first few layers it would seem transparent. Hence the principle formulated earlier (Mina, 1968) for fish otoliths also applies to the dentine of mammals: visual estimates of both "transparent" and "opaque" layers are not absolute but relative. It is clear that when searching for the cause of changes in the optical density of dentine layers either the optical density should not be estimated visually at all, or else only adjacent layers should be compared. p. 1409

The effect of section thickness on the structure of the GLG. When the thickness of the section is reduced, the optical density of all its parts is reduced. Furthermore, the tendency for the overall transparency of the dentine to increase from the periphery of the section to the centre of the tooth becomes more pronounced and the structure of the GLG shows more clearly (Fig. 1, I). For example, in Tursiops truncatus the layers are difficult to differentiate on the curves from sections with a thickness of 300 μm and easy to distinguish on sections with thicknesses of 100, 80, 70 and 40 μm. It should be noted that in transmitted light the layers are hard to see in sections with a thickness of 300 μm, easy to see in sections with the thicknesses ranging from 100 to 70 μm, and far harder to see in thin sections of 40 μm. In fur seals the structure of the first few GLG of dentine is clearly visible both on the sections and on the characteristic curves at section thicknesses of 150 and 75 μm, but imperceptible even on such a curve at a section thickness of 40 μm (Fig. 1, II). The minimum section thickness at which the structure of the GLG is still visible is probably subject to individual variation.

By no means always but often, when the section thickness is reduced the structure of the GLG appears to change: the optical density curve reveals additional maxima, previously existing maxima appear to be displaced, and in some cases the relative optical density of part of the section may alter. For example, in Fig. 1, I it can be seen that on the characteristic curve of the section 80 μm thick the following changes have occurred compared with the curve of the section 150 μm thick: while retaining their general configuration curve segments 1-2' and 3-3' have shifted somewhat towards the periphery, segment 2a has altered its appearance, a new maximum 2a' has appeared, segments 4-5 have disappeared and been replaced by a segment in which the transparency increases continuously, and segments 6-7 have become so transparent that they are no longer on the scale of the KSP-4 recorder.

The change in the structure of the GLG when the thickness of the section is reduced is probably due to the fact that the layers of dentine have a definite curvature relative to both the transverse and the longitudinal axis of the tooth, so that when the thickness of the section alters the effect of the underlying layers on the optical density of the part of the tooth, in question changes. J. Mead (oral communication) has suggested a simple model demonstrating this effect. Let us assume that the GLG consists of a broad opaque layer, a narrow transparent one, a narrow opaque one and a narrow transparent one. When a thick section is examined in transmitted light the projection of some of the layers on the others may obscure the narrow opaque layer which is so to speak concealed by the adjacent transparent layers. The GLG will then appear to consist of 1 opaque and 1 transparent layer. When a very thin section is examined in transmitted light the broad opaque band may

disappear if the contrast between it and the transparent one is less than between the narrow opaque layer and the transparent ones. The GLG will then appear to be made up of a broad transparent layer and a narrow opaque one. Very likely something similar happened in our case as well. It follows that when studying the factors causing the differences in optical density of dentine layers the section should be of the minimum thickness at which the structure of the GLG can still be seen.

The effect of clearing agents on the structure of the GLG. When thin (30-80 μm) sections are mounted in glycerine there is a sharp increase in the transparency of the entire section and the characteristic curve shifts into the region of high transparency. In some cases, such as that of the fur seal (Fig. 2, I), the structural details of individual GLG are easier to see in a cleared section, the contrast of the main layers of the GLG remains the same and their arrangement remains unaltered. In other cases, such as that of Tursiops truncatus (Fig. 2, II), the same thing happens as when the thickness of a section is reduced: the relative optical density of certain areas in the root changes and there p. 1411 is a corresponding change in the structure of the GLG (see peaks 1a-5, Fig. 2, II). As is known, when specimens are mounted in a medium with a low index of refraction more detail can be seen even without staining, since the difference between the refractive indices of the tissue and the mounting medium is then great (Lilly, 1969). Since the refractive index of glycerine is low (1.45), this appears to be the reason why extra structural detail can be seen and why changes occur in the relative optical density of some areas in sections after they have been immersed in glycerine. It follows that when studying the effect of p. 1412

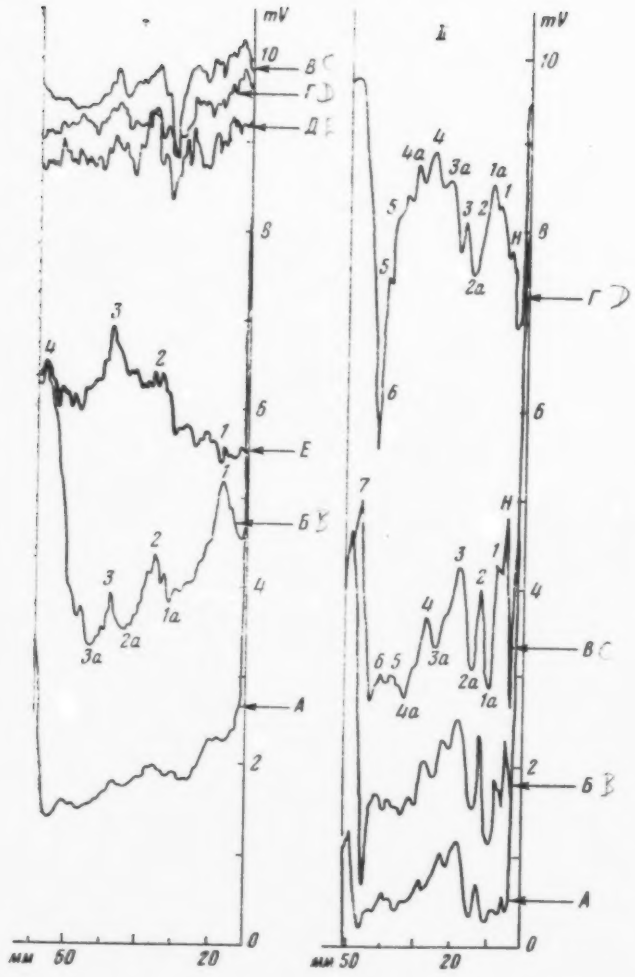

Fig. 2. Characteristic curves of the same area of a transverse section (thickness 30 μm) of a fur seal's tooth (I - ♀, 6 GLG) and a longitudinal section (different thickness) of the tooth of Tursiops truncatus (II - ♀, 7 GLG), lighting constant. I: A - dry section; B - section mounted in glycerine; C - same after the first decalcination, D - the same after the second decalcification, E - the same after the third decalcification, E section after the third decalcification, stained and mounted in glycerine. II: A - dry section 310 μm thick, B - 150 μm, C - 80 μm, D - section 80 μm thick mounted in glycerine. The same symbols as in Fig. 1 are used for identifying the layers

various processing methods on the structure of GLG we should compare sections which have been mounted in the same medium.

The effect of decalcification of dentine on the structure of GLG. Sections decalcified in a 5% solution of nitric acid were tested with sodium oxalate to determine the degree of decalcification (Lilly, 1969) and then mounted in glycerine, after which the optical density curve was compared with that of an undecalcified section mounted in glycerine. Both partial and full decalcification resulted in an increase in the transparency of the dentine, while the contrast of the individual layers was sharply reduced. Thus, the ratio of the height of the greatest maximum to that of the smallest minimum on the curve was reduced 1.5-2.1 times in Tursiops truncatus, 1.4 times in fur seal, and 2.1 times in the Caspian seal. In Tursiops truncatus the tendency for the transparency of the section to increase from the periphery to the centre of the tooth virtually disappeared after decalcification, whereas in Caspian seal the reverse tendency - for the transparency to diminish from the periphery to the centre of the tooth - became less pronounced.

In all the marine mammals investigated both complete and partial decalcification led to the appearance of previously invisible thin layers of varying optical density within the GLG (Fig. 2, I; 3, I), and the more complete the decalcification the more visible the thin layers were. In one of the Caspian seals examined the total optical density of the dentine was so great that the layers and boundaries of the GLG could not be seen on the optical density curve of a 50-μm-thick dry and a section mounted in glycerine, but were clearly visible after decalcification.

Hence we can conclude that the contrast of the main layers of the GLG and the tendency for the optical density of the dentine to alter from the periphery of the tooth to the centre are due mainly to the content of mineral component in the dentine, while the thin layers within the GLG are due to the structural features of the organic stroma of the tooth. Moreover it is probable that the contrast of the layers is influenced not only by the amount of mineral component but also by its distribution in the organic stroma.

The effect of collagen removal on the structure of the GLG. Collagen was removed by Schmidt's method (Romeis*, 1953) using sections 120-140 μm thick. After removal of the collagen the section was mounted in glycerine. It should be emphasized that this method is not simple and requires extreme care since the section can easily crumble after the collagen has been removed.

As a result of the removal of the collagen the overall transparency of the section is increased, the general shape of the characteristic curve remains virtually unchanged, the position of some peaks remains the same as before whereas that of others is slightly altered, and no fine layers can be seen (Fig. 3, II). On the whole the contrast of adjacent layers remains the same as before. For example, on one section from a fur seal consisting of 6 pairs of layers, 5 retained their former contrast. On another section from the same animal the contrast of a pair of layers situated fairly close to the centre of the section changed, whereas it remained unchanged between the other pairs of layers. It should be noted that change in the contrast of the

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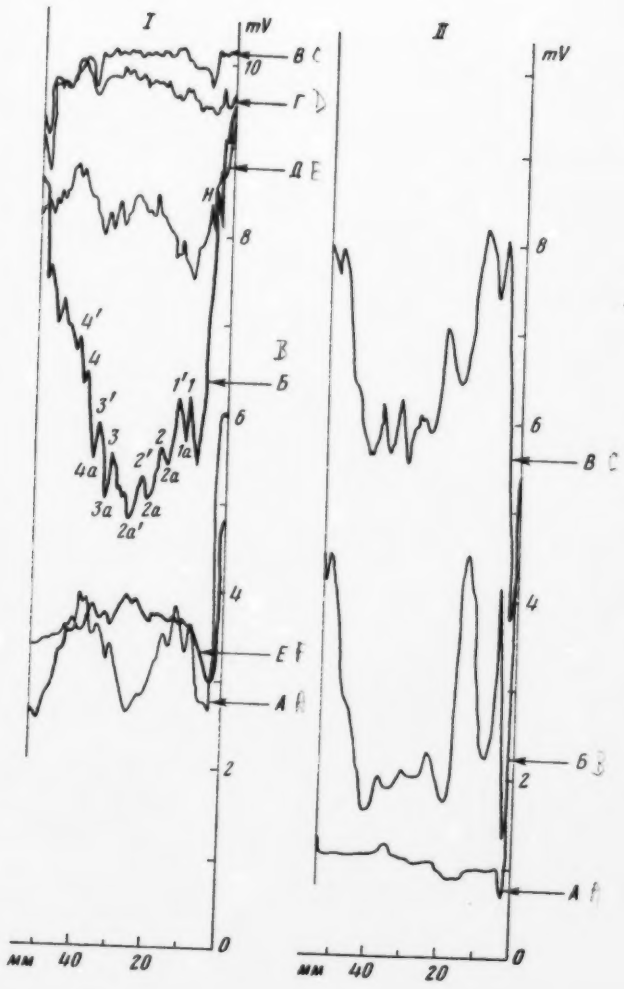

Fig. 3. Characteristic curves of the same part of a transverse section of a Tursiops truncatus tooth (I - ♀, 4 GLG) and the tooth of a fur seal (II - ♀, 6 GLG), lighting constant. I: thickness of section 30 μm; A - dry section, B - section mounted in glycerine, C - the same after the first decalcification, D - the same after the second decalcification, E - the same after the third decalcification, E - the section after the third decalcification stained and mounted in glycerine. II: thickness of section 140 μm; A - dry section, B - section mounted in glycerine, C - section after removal of collagen, mounted in glycerine. Designations of layers as in Fig. 1.

extreme layers (in one case peripheral, in the other central) is unsystematic and is probably due to the destruction of those layers during the preparation of the specimens by this particular method. After the collagen has been removed mineral globules can be clearly seen in the dentine. In the fur seal the more transparent layers consisted of larger globules, while the less transparent ones included small globules and star-shaped granules.

On the whole the data obtained confirmed the conclusions made in the previous section: the contrast of the main layers of the GLG is due to the mineral component of the dentine, while the thin layering is associated with the structural features of its organic stroma.

S t a i n i n g o f d e n t i n e w i t h E h r l i c h ' s h a e m a t o x y l i n. Sections 30-50 μm thick were partially decalcified, washed in water, stained with a mature solution of Ehrlich's haematoxylin and then mounted in glycerine. After the characteristic curve have been recorded the glycerine was washed off, decalcification completed (with the haematoxylin being removed from the dentine), the section was washed in water and stained again with haematoxylin. Staining lasted for the same period of time on both the first and the second occasions. In a number of cases the section was stained only after complete decalcification.

An incompletely decalcified section stains less intensely with haematoxylin than one which is completely decalcified, but in both cases the staining is uneven, i.e. some areas are more intensely stained than others. On the whole the areas which were more transparent prior to decalcification stained the most intensely. For example, in <u>Tursiops truncatus</u> (Fig. 3, <u>I</u>) the middle part of the section which

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was less transparent prior to decalcification stained somewhat less intensely than the peripheral and central parts. A comparison of the individual layers reveal the same thing: the less transparent layers GLG-3, GLG-2 stained less intensely than the more transparent GLG-1, GLG-4. The same applied to the fur seal sections as well (Fig. 2, I): on an undecalcified section there was a tendency for the transparency to diminish from GLG-1 to GLG-3. On the stained section there was a matching tendency for the intensity of staining to diminish: the transparent GLG-1 stained far more intensely than the less transparent GLG-2 and GLG-3. However the same curves may display the opposite tendency as well: in the last GLG of a fur seal tooth the curves recorded before and after staining coincide completely, i.e. the less transparent layers of dentine stained more intensely in this part of the tooth.

The contrast of individual layers brought out by staining was less than on an undecalcified section, but slightly higher than on a decalcified one. For example, in Tursiops truncatus the ratio of the optical density of the most weakly stained layer to that of the most intensely stained one was 1.4, the ratio of the transparency of the most transparent layer to the most opaque layer on an undecalcified section embedded in glycerine was 1.6, and on a decalcified section 1.05. The corresponding ratios for the fur seal were 1.24, 1.6 and 1.01 respectively.

The data obtained show that the intensity of staining with haematoxylin depends on the content of calcium in the particular part of the dentine, and that the layers of dentine which were most transparent prior to decalcification stained more intensely with haematoxylin on the whole. However, in some cases the pattern may be disturbed, to the

extent that the opposite tendency is exhibited. It is unclear why this should be so. As a rule the neonatal line does not stain.

It is believed that those areas of dentine which contain more mineral matter prior to decalcification stain more intensely with haematoxylin after decalcification (Schour, Massler, 1949). If this is so, our data provide grounds for assuming that as a rule the areas of dentine which are more transparent contain more mineral matter than those which are less transparent.

X - r a y s p e c t r a l a n a l y s i s. The distribution of the calcium and protein component in the layers of dentine was recorded as a function of the intensity of the characteristic emission of calcium CaK_{α} and sulfur SK_{α} contained in the proteins of the dentine.

Before the X-ray spectral analysis we prepared a transverse section of a Tursiops truncatus tooth (5 year old female) 330 μm thick. After analysis the specimen was ground (on the side opposite to that analyzed) to a thickness of 60 μm and then a microphotometer was used to obtain a curve of the changes in optical density along a line as close as possible to the X-ray spectral analysis line.

The qualitative-quantitative curves characterizing the intensity of emission of calcium and sulphur were displaced compared with the curves characterizing change in optical density. It was found that there was a tendency for the quantity of calcium to increase from the periphery to the centre of the tooth, which corresponds to an overall increase in the transparency of the section in the same direction. The areas of relatively greater transparency on the optical density curve corresponded to the areas of increased calcium concentration, while the areas of relatively lower transparency corresponded to areas of reduced

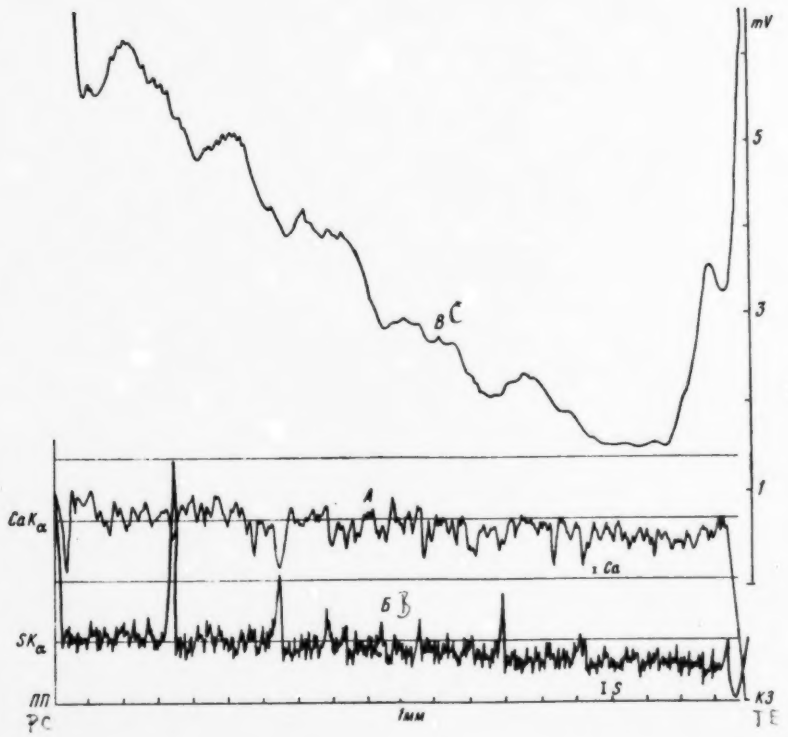

Fig. 4. X-ray spectral curves of the intensity of Ca and S emission and the optical density curve of the same area of a transverse section of a Tursiops truncatus tooth (♀, 5 GLG) on the same scale: A - curve of Ca emission intensity (CaK_α); B - curve of S emission intensity (SK_α); C - characteristic curve; Ca - level of noise of calcium; S - level of noise of sulfur; PC - pulp cavity; TE - edge of tooth

calcium concentration on the curve characterizing the intensity of calcium emission (Fig. 4). The exception is the first area of increased transparency corresponding to the neonatal line which has the same calcium content as the adjacent areas.

The curve characterizing the intensity of sulfur emission displays sulfur content maxima and minima which correspond to the areas where the calcium concentration diminishes and increases sharply. In this respect our data coincide with those of Aps et alia (1982) who

noted that the changes in the content of calcium and sulfur for otoliths were strictly opposite in phase. In some cases the areas of increased sulfur concentration correspond to the presumed boundary of the GLG (Fig. 4).

Hence, X-ray spectroanalysis of the Tursiops truncatus section shows that the areas of dentine which are relatively more transparent contain relatively more mineral component than the areas which are relatively less transparent.

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Summary

The causes of formation of the dentine layers were studied by microphotometry and X-ray spectral analysis. The visual estimate of the dentine layer optical density was shown to depend on the optical density of adjacent layers, i. e. it is relative. The optical characteristics of layers in the growth layers group (GLG) can alter both with the change of section thickness and with the section embedding into a clearing medium. The optical density of the main layers within GLG appears to be determined by the content and distribution of a mineral component of dentine. More transparent zones contain, as a rule, more mineral substances than less transparent ones. The neonatal line appears to be, however, not hypercalcified and its transparency is due to the other causes. The lesser layers within GLG are determined by differences in the structure of tooth organic stroma.